Reservoir and vector evolutionary pressures shaped the adaptation of *Borrelia*.

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Abstract

The life cycle of spirochetes of the genus *Borrelia* includes complex networks of vertebrates and ticks. The tripartite association of *Borrelia*–vertebrate–tick has proved ecologically successful for these bacteria, which have become some of the most prominent tick-borne pathogens in the northern hemisphere. To keep evolutionary pace with its double-host life history, *Borrelia* must adapt to the evolutionary pressures exerted by both sets of hosts. In this review, we attempt to reconcile functional, phylogenetic, and ecological perspectives to propose a coherent scenario of *Borrelia* evolution. Available empirical information supports that the association of *Borrelia* with ticks is very old. The major split between the tick families Argasidae–Ixodidae (dated some 230–290 Mya) resulted in most relapsing fever (Rf) species being restricted to Argasidae and few associated with Ixodidae. A further key event produced the diversification of the Lyme borreliosis (Lb) species: the radiation of ticks of the genus *Ixodes* from the primitive stock of Ixodidae (around 217 Mya). The ecological interactions of *Borrelia* demonstrate that Argasidae-transmitted Rf species remain restricted to small niches of one tick species and few vertebrates. The evolutionary pressures on this group are consequently low, and speciation processes seem to be driven by geographical isolation. In contrast to Rf, Lb species circulate in nested networks of dozens of tick species and hundreds of vertebrate species. This greater variety confers a remarkably variable pool of evolutionary pressures, resulting in large speciation of the Lb group, where different species adapt to circulate through different groups of vertebrates. Available data, based on *ospA* and multilocus sequence typing (including eight concatenated in-house genes) phylogenetic trees, suggest that ticks could constitute a secondary bottleneck that contributes to Lb specialization. Both sets of adaptive pressures contribute to the resilience of highly adaptable meta-populations of bacteria.

Keywords: *Borrelia*; evolutionary pressure; tick-*Borrelia*-reservoir interaction

Highlights

- Ancestral *Borrelia* could be a primitive symbiont that led to relapsing fever spp.
- Lyme borreliosis species diversified after the *Ixodes* genus split.
- Lb species lost vertical passage capacity after transmission through reservoirs.
- Disparate Argasidae/Ixodidae life cycles favoured differential *Borrelia* evolution.
- Phylogenetic analyses suggest selection pressure on Lb by tick vectors.

1. Introduction

The genus *Borrelia* is a large assemblage of bacterial species that has gained a great deal of attention in the recent decades because of their importance for human health in the northern hemisphere (Burgdorfer et al., 1982; Lane et al., 1991; Steere et al., 1983). Studies have demonstrated that *Borrelia* is phylogenetically related to the genera *Treponema*, *Brachyspira*, and *Leptospira*, which include not only important pathogens but also free-living and non-pathogenic bacteria that can colonize the midgut of Arthropoda (Loh et al., 2017). These genera belong to the family Spirochaetaceae that also includes the genera *Clevelandina*, *Cristispira*, *Diplocalyx*, *Hollandina*, *Pillotina*, *Spirochaeta*, and *Sphaerochaeta* (Paster, 2011; Euzéby, 2013).

Infected ticks can transmit *Borrelia* to a large group of vertebrates (e.g., reptiles, birds, and small mammals). *Borrelia* adapted to this dual vertebrate–tick life cycle, which is crucial for the persistence of natural foci. There are two major groups of species of *Borrelia*, namely relapsing fever (Rf)[1] and Lyme borreliosis (Lb). The *Borrelia* spp. included in the Rf group are commonly transmitted by ticks of the family Argasidae, but some species of Ixodidae also may serve as vectors. The species included in the Lb group, also known as the "*Borrelia burgdorferi* sensu lato complex of species", are transmitted exclusively by a few species of ticks within the genus *Ixodes*. All species of *Borrelia* have a transstadial transmission, i.e., the pathogen survives moulting within the vector from one life stage to the next. In addition, Rf species show transovarial transmission, i.e., the pathogen is passed from female ticks to the offspring. The latter has never been recorded in Lb species. This transovarial (also called vertical) transmission in the tick, however, produces only low and variable rates of infection in the progeny (Barbour and Hayes, 1986). Based on the relatively low transovarial transmission rates, it has been proposed that the temporal transmission to vertebrates evolved as an 'amplifying force', allowing the bacteria to persist in natural foci (Tsao, 2009).

After the characterization of the genus *Borrelia*, new Rf species were described, associated with different vectors (Cutler et al., 2017). The species were collectively considered under the rather arbitrary groupings of Old World and New World borreliae, using the prevailing concept at that time of "one species–one vector" (Cutler et al., 2017). The discovery of *B. burgdorferi* s.l., now considered to be a large complex of 21 species, and its association with humans as the etiological agent of the Lyme borreliosis (Steere et al., 1983), promoted a reconsideration of the complexity of the genus. At that time, the partial availability of data on the epidemiological cycle of each species resulted in the conviction that the Rf species were transmitted only by argasid ticks, and Lb species

[1] Abbreviations: Rf, and LB: relapsing fever or Lyme borreliosis groups of species of Borrelia, respectively; osp: outer surface protein

by ticks of the family Ixodidae (except for the human louse-borne *Borrelia recurrentis*). This dogma held for many years, but new Rf species transmitted by ixodid ticks were discovered, such as *B. miyamotoi*, *B. lonestari*, and *Candidatus* "B. texasensis" (Fukunaga and Koreki, 1995; Lin et al., 2005; Varela et al., 2004), requiring its reconsideration. These species are believed to use what are regarded as "sub-niches" of the vertebrates in which some Lb species thrive. It is still necessary to study how the *ecological* relationships of *Borrelia* could explain the evolutionary pressures that promote the different molecular machinery of each group.

This review is not devoted to revisiting the molecular peculiarities of *Borrelia*. Excellent reviews on this topic are available elsewhere (i.e., Anguita et al., 2003; de Taeye et al., 2012; Kung et al., 2013; Pal and Fikrig, 2003; Schwan and Piesman, 2002; Tsao, 2009). Instead, we aimed to use the available empirical grounds to understand the diverse evolutionary pressures that may have influenced the evolution of both groups of *Borrelia*, Rf and Lb, proposing an evolutionary line associated with geological and tick evolutionary events. We review the similarities in some of the molecular mechanisms *Borrelia* use to survive in either the tick or the vertebrate. We hypothesize that the large set of biotic interactions of Lb species with ticks and vertebrates explains the genetic variability of this group compared to Rf. We further analyse available molecular data and conclude that tick-derived evolutionary pressures have a critical impact in the evolution of *Borrelia*.

2. Molecular machinery of *Borrelia* spp.

Recent research using genomics, transcriptomics, and proteomics has provided unprecedented knowledge of the molecular mechanisms that borreliae use to survive in the tick and vertebrate hosts. Infection and colonization of such a disparate set of hosts pose major challenges to these bacteria. First, the bacteria must escape and survive invertebrate and vertebrate immune systems (Hajdušek et al., 2013; Kurtenbach et al., 2002). Second, within the tick midgut, they have to overcome toxic components resulting from tick blood digestion and anti-microbial peptides (Abraham et al., 2016). Third, *Borrelia* must survive the breakdown of tissues associated with tick moulting. To face these challenges, *Borrelia* have evolved quite interesting molecular strategies. The genome of *Borrelia* consists of one chromosome and a variable number of plasmids that represent up to 40% of the genome (Grimm et al., 2005). *Borrelia* is the bacterial genus in which the largest number of plasmids has been reported (Casjens et al., 2000), allowing ample exchange of genetic material within and between species (Grimm et al., 2005). Studies suggest that many plasmid genes encode proteins important to borreliae persistence in various vertebrates (Barbour and Garon, 1987). Gene composition in the chromosome is highly conserved among *Borrelia* species, but the genetic information in the plasmids varies greatly among species and strains within a species (Grimm et al., 2005). Lipoproteins (e.g., the outer surface proteins, or osp) play an

117 important role in the molecular mechanisms of adaptation of these bacteria to both the tick and the
118 vertebrates.

119 *2.1. Entering the tick with the blood meal*

120 The process of blood digestion in ticks greatly differs from that in insects. In ticks, digestion is a
121 slow intracellular process (Arthur, 1965; Balashov, 1972). With few exceptions, Argasidae feed
122 quickly and several times (around 40–60 minutes per feeding). The virgin females of Argasidae
123 start digesting their blood meal, but blood digestion does not proceed further than initial stages until
124 mating occurs. Virgin females of Ixodid metastriate ticks take only a small quantity of blood before
125 mating (Sonenshine, 1991). The unmated females will remain attached to the host for several weeks
126 without feeding much more. When mating occurs, the female will imbibe a huge meal, increasing
127 its weight approximately 100 times in a very short time (Sonenshine, 1991). The more primitive
128 prostriate *Ixodes* ticks, however, may copulate both in the absence of hosts and while the female
129 engorges. In these case, autogenous spermatogenesis must precede host contact (Kiszewski et al.,
130 2001).

131 The Rf species enter the tick very quickly and can be detected in the gut lumen a few minutes after
132 the beginning of the attachment of Argasidae (Schwan and Piesman, 2002). Because of the tick's
133 fast feeding, a rapid transmission from the vertebrates is necessary to ensure a successful
134 acquisition. Probably this is why Rf species are acquired by ticks only during a certain level of
135 spirochetemia (Lopez et al., 2011). The Lb species can be detected in the midgut of the tick within
136 the first 24 hours of attachment, well before significant amounts of blood have been imbibed
137 (Schwan and Piesman, 2002). In both argasid and ixodid ticks, the first step of blood digestion is
138 concentration of the blood meal by elimination of the excess water. Ixodid ticks use their salivary
139 glands to concentrate the water and shuttle it back to the host, whilst argasids use their coxal organs
140 to excrete the excess of water out of the tick (Sonenshine, 1991). The concentration of the blood,
141 together with the proteolytic activity in the gut lumen, could be responsible for the clearance of the
142 bacteria from the gut and the low number of *Borrelia* in the next stage of the tick. Most bacteria
143 could be wiped out of the gut, so this step seems to be a critical part of the life cycle of *Borrelia*
144 inside the tick (Tsao, 2009). However, the low number of borreliae in the next tick stage could also
145 result from the proteolytic activity associated with tick moulting. As far as we know, no empirical
146 evidence exists explaining how borreliae overcomes these processes.

147 It is currently accepted that after a brief colonization of the tick gut, every Rf species quickly
148 migrates to the salivary gland of the argasid vectors (Schwan and Piesman, 2002). This rapidity
149 may explain the fast transmission times of Rf species from their tick vectors to the vertebrate. It has
150 been demonstrated that *Borrelia turicatae* is transmitted by *Ornithodoros turicata* by about 15

seconds after attachment (Boyle et al., 2014) and then remains in the body of starved ticks for years (Schwan and Piesman, 2002). The Lb species, transmitted by ixodid ticks, display a complex behaviour leading to the transcription of some proteins, believed to "anchor" the Lb species to the tick gut and preventing the immediate passage to the salivary gland. Only after moulting does a new blood feeding trigger migration of Lb species from the tick gut to the haemocoel and salivary glands for transmission into the vertebrate. This distinction seems to be more related to genetic differences between the two groups of *Borrelia* than to the particularities of the physiology of ixodid and argasid ticks. For example, *B. miyamotoi*, an Rf transmitted by Ixodidae, follows the same route as other Rf species. This species can be found in the salivary gland of the next, newly moulted stage, even without the compulsory blood meal necessary to trigger its migration necessary for Lb species (Barbour, 2014).

The Lb species remain in the tick gut for the complete moulting period without migrating to the salivary glands. In some ticks transmitting *Borrelia*, this period may last for months. By the time the ixodid tick is fully engorged, Lb species have upregulated a series of genes specifically expressed in the tick environment. Among these, perhaps the most studied is *ospA*, which is co-expressed with *ospB*, and both are encoded in the linear plasmid lp54 (Barbour and Garon, 1987). It has been proposed that host neuroendocrine stress hormones produced in the skin of hosts in response to tick attachment bind Lb species, inducing *ospA* expression (Scheckelhoff et al., 2007). In consequence, once Lb species access the tick gut, *ospA* and *ospB* are already upregulated. OspA and ospB are functionally equivalent, anchoring the borreliae to the tick gut cells (Tilly et al., 2016). After moulting, when ticks take a new blood meal, the expression of *ospA* and *ospB* is downregulated and *ospC* is upregulated.

In addition to lipoproteins, the Hk1-Rrp1 pathways, present in both Rf and Lb, are also required for successful colonization of the tick midgut. At least in the Lb species, the Hk1-Rrp1–deficient mutants are cleared during the tick feeding because they cannot protect themselves against antimicrobial products of the tick gut (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). The fact that the Hk1-Rrp1 pathway components are present in both Rf and Lb suggests that this is a primitive molecular machinery that could be present in the common ancestor of both groups of *Borrelia*. The system Hk1-Rrp1 potentially constitutes an essential system for the survival of borreliae during larval and nymphal tick blood meals. Lb species deficient in either protein are virulent in vertebrates but are killed within the tick gut soon after feeding (Caimano et al., 2011; He et al., 2011; Kostick et al., 2011). Of interest, Lb species over-expressing Rrp1 are non-infective for mice (Kostick et al., 2011), suggesting that, similar to osp proteins, Rrp1 should be differentially

184 regulated during the life cycle of *Borrelia*. For a comprehensive list of the currently identified
185 proteins necessary for the survival of Lb species in ticks, see Kung et al. (2013).

186 The evidence suggests that the tick gut is a hostile environment for the survival of borreliae,
187 probably because of the production of antimicrobial peptides with borreliacidal activity (Sonenshine
188 et al., 2005). A defensin-like peptide is upregulated in *Ixodes* ticks after infection with *B.*
189 *burgdorferi* (Rudenko et al., 2005), together with the expression of some other genes potentially
190 involved in the oxidative stress response. In addition, blood digestion products appear to be toxic
191 for *Borrelia*. Tick microbiota can also affect the survival of *Borrelia* in the tick gut (Narasimhan et
192 al., 2014). It has been proposed that the function of ospA-ospB is to protect Lb species from host-
193 derived bactericidal antibodies in the feeding tick gut (Battisti et al., 2008). However, Tsao (2009)
194 proposed that the "retention" of Lb in the tick gut has an epidemiological role and that the bacteria
195 access the tick salivary glands only when ticks start to feed. The timing of Lb migration to the
196 salivary gland would be critical for successful transmission to the vertebrate.

197 *2.2. Infection of the vertebrate host and evasion of the immune system*

198 All Rf species (including those associated to Ixodidae) access the salivary glands soon after being
199 acquired within the blood and therefore are ready to be inoculated into the vertebrate as soon as the
200 tick begins to feed. There is a possibility that the reports of Lb transmission within a few hours after
201 tick feeding starts may refer to *B. miyamotoi* (Rf) and not to Lb (Barbour, 2014; Cutler et al., 2017).
202 The ecological inferences from observations on argasids are difficult to apply to Rf species
203 transmitted by Ixodidae because these ticks will enter a long period of moulting and the subsequent
204 feeding stage will last for days. The ecological necessity of a fast migration of the Rf species to the
205 salivary glands cannot be explained as an epidemiological trait driven only by the ecology of the
206 tick, and probably is an ancestral trait of the primitive borreliae that has been conserved during
207 evolution.

208 As mentioned above, *ospC* is upregulated in Lb species only when a new blood intake occurs in
209 Ixodidae. It was initially postulated that changes in tick gut temperature and pH during feeding
210 promote the downregulation of *ospA-ospB* and the upregulation of *ospC* (Ramamoorthi and Scholl-
211 Meeker, 2001). However, transcriptional regulation of these genes do not occur *in vitro* when
212 cultures of Lb species are exposed to different temperatures and/or pH. Thus, other components of
213 the blood may trigger the transcriptional regulation of these proteins. OspC binds plasminogen to
214 support the migration of the borreliae across the tick body (Coleman et al., 1997; Lagal et al., 2006;
215 Önder et al., 2012).

216 The borreliae need to circumvent recognition by the vertebrate complement during and after
217 transmission by the tick. The complement system is a complex network of proteins activated in a

cascade. The main force driving speciation of the Lb species is the ability to evade the sera of different groups of mammals, birds, or reptiles (Kurtenbach et al., 2002). The complement of the species of vertebrates that can circulate a species of *Borrelia* cannot produce the complement membrane attack component (MAC) against these bacteria. For a complete description of borreliae inhibition of the three pathways of the vertebrate complement, see de Taeye et al. (2012). It is now well-established that a tick protein present in the salivary glands, Salp15, selectively binds ospC (Anderson and Valenzuela, 2007; Hovius et al., 2008; Narasimhan et al., 2007; Ramamoorthi et al., 2005). This protein inhibits T-cell activation (Anguita et al., 2002) and is selectively increased in *B. burgdorferi*–infected tick salivary glands during the engorgement of *I. scapularis* (Ramamoorthi et al., 2015). Other proteins, Salp20 and Salp25D, have been identified in other species of *Ixodes* (Tyson et al., 2007). This family of proteins has now been described in several tick genera as blocking the activation of host alternative complement pathway and preventing the formation of reactive oxygen species (Das et al., 2001). The selective binding ospC-salp15 must be regarded as the exploitation of existing tick immunosuppressive mechanisms by the borreliae. The bacteria also take indirect advantage of tick salivary proteins other than salp15 that inhibit complement activation at the tick bite site; see the following comprehensive studies for an overview (Couvreur et al., 2008; Das et al., 2001; Gillespie et al., 2001; Lawrie et al., 1999, 2005; Nuttall, 1999; Schuijt et al., 2011a, b; Tyson et al., 2007, 2008).

After entry into a competent vertebrate, persistent infection must be established by evading the immune system through an antigenic variability. In Rf species, this step is accomplished mainly by the variable major proteins (Vmp). These Vmps occur in two size classes: the variable large proteins (Vlps) and the variable small proteins (Vsps). As reported by Schwan and Piesman (2002), a single cell of *Borrelia hermsii* can give rise to 30 antigenic variants, each of which expresses a unique Vmp that confers a specific serotype (Barbour and Restrepo, 2000; Stoenner et al., 1982). One interesting finding is that both Rf and Lb species share a similar mechanism: the Vsp33 of Rf species is homologous to the ospC expressed by Lb species (Carter et al., 1994; Marconi et al., 1993; Margolis et al., 1994; Schwan and Piesman, 2002; Wilske et al., 1993). The Vsps are expressed by Rf to evade the immune response of the vertebrate by inhibiting CD4+T cell activation. These findings point to the conclusion that the Vsp of the Rf group is phylogenetically related to the ospC of the Lb group and that both are involved in the transmission from tick to vertebrate hosts.

3. The probable scenario of emergence and evolution of *Borrelia*

The data presented suggest a scenario for the evolution of *Borrelia*. Available findings support the conclusion that *Borrelia* is a relative of other spirochetes associated with Arthropoda (i.e., Gupta et al., 2013). Our arguments for the probable evolution of *Borrelia* spp. are as follows: (i) borreliae are associated with the primitive stock of ticks before the Argasidae–Ixodidae split; (ii) Rf species are the most primitive and remained mainly associated with argasid ticks after the Argasidae–Ixodidae split; (iii) Lb species derived from the primitive Rf group after the split of the prostriate group of ticks (Ixodidae) before the complete separation of land masses; and (iv) the evolution of the Lb species results from its association with the *Ixodes ricinus* group and the interactions with their vertebrate.

3.1. The evolution of ticks and its role in the speciation of primitive Borrelia

We adhere to Mans et al. (2016) as the most robust phylogenetic reconstruction of tick evolution. According to their hypothesis, ticks most probably originated in what is now known as Australia or Southern Africa, and then spread about 390 million of years ago (Mya). These estimations are based on mitochondrial DNA clocks. Following the same approach, the Ixodidae–Argasidae split has been calculated as having taken place about 290 Mya (Figure 1). The separation of the main lineages of *Ixodes* is estimated to have occurred about 217 Mya. Data concur that the Ixodidae–Argasidae split took place between the early Permian and early Triassic periods. The supercontinents Gondwana and Pangaea were colonized by ticks, with further speciation processes. The speciation of the stem classes of Mammalia took place ca. 235–205 Mya, and the oldest bird fossil dates to only 150 Mya (Mans et al., 2016).

We postulate an association of commensalism between primitive *Borrelia* and ancestral ticks that was established before the Ixodidae–Argasidae split. Afterwards, *Borrelia* adapted to a transmission through a vertebrate. Many bacteria of the phylum Spirochaetes are symbiotically associated with Arthropods (Berlanga et al., 2011). Studies of the microbiome of ticks indicate the presence of species of the phylum Spirochaetes (like *Treponema* and *Brevinema*) in the tick gut as part of the normal microbiome of ticks (our unpublished data). Except for a few Rf species transmitted by Ixodidae and *B. recurrentis*, transmitted by the louse, all Rf species are exclusively transmitted by argasid ticks (Cutler et al., 2017). There is a univocal association between each Rf species and the species of Argasidae that transmit them (Cutler et al., 2017) that does not exist in the Lb species. This pattern suggests adaptation (and possible speciation) of Rf species to their tick vectors that is older than the associations of Lb species and *Ixodes*. According to the well-supported phylogenies of the genus *Borrelia*, based on the multi-locus sequence typing scheme (MLST), Rf species are basal to the rest of the phylogenetic tree of the genus (Supplementary material 1). Most Rf species are transmitted from the female tick to the offspring. Exceptions are the Rf *B. hermsii* transmitted

by *Ornithodoros hermsi*, and *B. duttonni* transmitted by *O. moubata*, in which the vertical transmission is rare (Schwan and Piesman, 2002). Vertical transmission is also present in the Rf *B. miyamotoi*, transmitted by *Ixodes* ticks. On the other hand, all Lb species lack vertical transmission. We speculate that vertical transmission could be an ancestral feature of Rf species (lost in some Rf lineages, and, as far as it is known, also absent in every Lb species).

3.2. The adaptation of Borrelia *to ticks*

All extant Lb species express the same proteins interacting with the tick gut. Of special interest are the ospA-ospB proteins. Margos et al. (2009) demonstrated that the gene expressing ospA exists only in Lb species. It is more parsimonious to consider that the borreliae were associated with ticks before its split than to consider two different events of association, one for the Rf group and another for the Lb group. This hypothesis also is supported by the fact that a few species of the Rf group are associated with ixodid ticks. The ospA-ospB proteins should be present in the primitive Lb stock, before the speciation of the primitive *Ixodes*, because now they appear in every Lb species independently of the species of vector. Figure 2 includes the summarized lineages of Rf and Lb species based on *16S rRNA* sequences available in GenBank (see also Supplementary material 2 for the complete phylogenetic tree). The topic, however, warrants additional research because some "intermediate" species of *Borrelia* (e.g., lying out of the two main clusters of Rf and Lb) have been reported as associated with ticks of the genus *Hyalomma* (*B. turcica*), to the Australian *Bothriocroton* ("*Candidatus* B. tachyglossi) or to *Amblyomma geoemydae*, also associated to reptiles (Kalmár et al., 2015; Loh et al., 2017; Takano et al., 2011, 2012). Probably *B. turcica* is the best characterized species of the group above. However, only data regarding its transstadial transmission in the tick (Kalmár et al., 2015), its finding in blood of imported tortoises and the molecular characterization of *16S* and *gyrB* (Takano et al., 2011) have been carried out. The studies by Kalmár et al. (2015) and those by Takano et al. (2012) place the reptile-associated Borrelia near the Rf group but in a branch slightly separated of the main clade. Further elaboration on the topic would be purely speculative until new empirical data is available. There is thus an ample field of research on *B. turcica* and other species associated to ticks parasites of reptiles.

The species of Lb for which empirical evidence exists exhibit similar strategies for exploiting the saliva of the tick. Probably, the best characterized interaction is ospC binding with Salp15 produced by the salivary glands of the tick. The infection of the tick by Lb species upregulates Salp15 (Ramamoorthi et al., 2005), suggesting a manipulation of a tick protein that is older than the association of ticks and Lb species. Even considering the variability in *ospC* sequences (Gatewood et al., 2009; Qiu et al., 2008), the mechanism is the same for every Lb species for which evidence exists, supporting the hypothesis of only one original stock of Lb species. Figure 3 includes the

summarized lineages of Lb species based on *ospC* and *ospA* (see also Supplementary material 3 for the complete tree of sequences available on GenBank, which includes information on the species of tick). It is important to consider that the phylogenetic signal in *ospC* does not track either the species of Lb or the association with species of ticks. This has been reported to be the result of a horizontal gene transfer between species of *Borrelia* because the locus *ospC* is in a plasmid (Lin et al., 2002). The phylogeny based on *ospC* does not overlap that generated by 16S rRNA, although it has been reported that both phylogenies are coherent (Attie et al., 2007; Qiu et al., 2004). However, the phylogenetic tree built on *ospA* sequences (Figure 3B) displays an association between the species of *Borrelia* and the tick vector, which we will elaborate in the next section. It is interesting to note that recent evidence (Rudenko et al., 2016) indicates that some rare *ospC* types may circulate among non-*Ixodes* ticks in southeastern USA. Rudenko et al. (2016) proposed a separation or overlapping transmission cycles of *ospC* strains not restricted to *Ixodes* ticks and rodents.

3.3. The adaptation of Borrelia *to vertebrates*

It has been acknowledged that the transmission rates of extant borreliae to the eggs of the ticks are very low, in the species in which it has been observed. These low rates would impair the persistence of the bacteria in foci in consecutive generations of ticks if they were the only mechanism of circulation (Tsao, 2009). We consider that the primitive *Borrelia*, or an extinct ancestor, managed to reach the salivary gland of the tick and then passed into the feeding lesion in a vertebrate. The circulation of the pathogen can be amplified by the non-systemic, or co-feeding transmission, representing a potential route by which infected ticks may infect naïve ticks (Randolph et al., 1996; Tsao, 2009) by recently deposited borreliae into the feeding lesion by an infected tick infecting another closely or subsequently feeding tick. The relative importance of the co-feeding transmission in the presence of systemic transmission in nature is unknown (Piesman and Happ, 2001).

Ticks of the family Argasidae are burrow dwellers, and there is no reason to believe that this behaviour was not present in the stock of primitive ticks (Mans et al., 2012). This factor is of special importance because the use of burrowing animals as hosts constitutes a relatively secure source of food for ticks living in the shelter. However, the population of argasid ticks in a burrow commonly consists of only a few dozen specimens at different stages (Vial et al., 2006). Therefore, ticks feeding on burrowing animals guaranteed the transmission of primitive *Borrelia* to the next tick stages because of the close co-occurrence of both ticks and burrowing vertebrates. If the persistence of the borreliae in the local population of ticks in a burrow was enhanced by the vertebrate transmission route (according to the ecological hypotheses elaborated by Tsao, 2009), the competing strains of borreliae transmitted exclusively by transovarial passage would be excluded from the genetic pool because of their lower fitness. Accordingly, vertebrate transmission is an

advantageous ecological strategy for *Borrelia*, forcing the loss of unused genes (Moran, 2002), such as those implicated in transovarial transmission, once the resilience of the borreliae is secured by transmission through the vertebrate bridge. These genes could be lost by species in which the vertebrate route is resilient to keep their circulation and retained by species in which encounters with vertebrates have low probabilities, like the argasid living in burrows seasonally occupied by vertebrates.

It is open to speculation whether the primitive borreliae could gain access to the host lesion *via* the tick coxal fluid or the saliva. Some investigations demonstrated that *B. duttonii* is transmitted by contamination of infected coxal fluid and tick bite (Burgdorfer, 1951). In addition, the mode of transmission varies with the stage of the tick: *O. moubata* nymphs transmit *B. duttonii* in the saliva, and adults transmit primarily via the coxal fluid (Burgdorfer, 1951). Nevertheless, because not every argasid produces coxal fluid while feeding, this mechanism may not to be universal for entry of Rf species into a vertebrate. For example, *Ornithodoros hermsi* (transmitting *B. hermsii*) produces coxal fluid off the host only after the feeding is complete (Schwan and Piesman, 2002). Furthermore, it has been demonstrated that some Rf species, like *B. coraciae*, do not leave the tick body *via* coxal fluid (Lane and Manweiler, 1988), whereas others copiously do (*B. johnsoni* in the bat tick *Carios keyelli*, Schwan et al., 2009). Thus, the coxal fluid does not seem to be a consistent route for transmission of Rf species.

A selective pressure on borreliae may have appeared when their primitive ancestors acquired abilities to evade the immune system of the vertebrates. Whereas proteins in argasid saliva modulate the host's immune response (e.g., Nunn et al., 2005), empirical evidence is lacking for Rf species exploiting these proteins, although the similarities between ospC and Vsp could provide evidence for Rf exploiting these proteins. The evolutionary pressure by the vertebrate can be hypothesized to be the origin of the Vlp and Vsp that Rf species use to evade the antibody response of the vertebrate. Both *vsp* and *vlp* genes are located in plasmids, which underpin antigenic variation during relapsing fever and are subjected to lateral transmission (Barbour, 2016).

3.4. Summarizing and consolidating the scenarios

We believe that enough proofs support the hypothesis that the Rf species persisted in ticks after the split into the two families, currently known to have occurred about 230–290 Mya (Mans et al., 2016). Therefore, the new lineage of Ixodidae already carried a set of primitive Rf species, which still widely persist in Argasidae and in some genera of Ixodidae. However, no Lb species are found in ticks of the family Argasidae, implying that events of speciation giving rise to Lb species occurred after the split of the genera of the family Ixodidae. Every Lb species investigated so far uses the same molecular strategies to colonize the tick gut and to enter the salivary glands and

exploit similar mechanisms of the tick saliva, gaining access to the vertebrate evading its immune system. Available data suggest that it is more plausible to consider the complete stock of Lb species evolving from existing Rf species, in a lineage of *Ixodes*, that further spread through Laurasia (Figure 1). Therefore, the split of Lb species should have occurred after the genus *Ixodes* separated from the rest of the ticks, something that is not dated before 217 Mya (Mans et al., 2016). That primitive Lb stock then evolved into different species according to regional environmental pressures, including particular adaptations to local prevailing vertebrates. The split of the genus *Ixodes*, the divergence of the mammaliaform clades, and the separation of the land masses of Laurasia are all concurrent in time and dated around 235–249 Mya (Mans et al., 2016). After Laurasia broke into the Palaearctic and Nearctic, Lb species became genetically isolated, excluding punctual population turnovers (Qiu et al., 2008). Our view is that the split between Rf and Lb is very old; therefore, it makes no sense to elaborate on a "European" or "American" origin of the Lb stock: the common ancestor appeared from an Rf stock *before* the land masses of Laurasia separated and then evolved under pressure of environmental traits and vertebrate availability. The movements of ticks and the carried Lb stock between land masses (like the east of Russia, Japan, and western Nearctic) or the variability of *B. burgdorferi* s.s. in both Nearctic and Palearctic (Qiu et al., 2008; Margos et al., 2012; Walter et al., 2017) require further research. The recording of a new species of the complex, *B. chilensis*, in the Neotropics (Ivanova et al., 2014) has a special interest considering that North and South America remained separated until about 3 Mya. The complete MLST phylogeny available in Supplementary Material 1 supports a basal phylogenetic position of *B. chilensis* regarding the other Lb species. Therefore, the species could have probably evolved earlier, when Afrotropical and Neotropical regions remained joined in one land mass. A complete sequencing of this species would provide important data regarding its proteome in comparison with other Lb species.

4. Uncovering the ecological relationships among ticks, *Borrelia*, and vertebrates

The analysis of the ecological relationships among ticks, vertebrates, and *Borrelia* spp. reveals the patterns underlying them. Previous studies (Estrada-Peña et al., 2015, 2017) applied the general framework of graph tenets to the recorded associations among the partners. The power of a network analysis lies in its ability to detect associations, thus uncovering ecological patterns that are otherwise difficult to identify. In addition, the networks summarize information on ecological relationships in clusters of interacting organisms for which the "strength of the interaction" can be measured. The network of the recorded ecological interactions among ticks, *Borrelia* spp., and vertebrates is included as Supplementary material 4 and 5. The network is a large improvement over

its original version (Estrada-Peña and de la Fuente, 2017; Estrada-Peña et al., 2017) because it has almost twice the number of records (>10,000) and includes both Rf and Lb species, as well as every species of tick and associated vertebrates.

Data in the network offer the most complete information about the ecological relationships of *Borrelia* and therefore of the framework on which evolutionary pressures act. It shows a holistic view of the community, with each group of organisms displaying the recorded ecological relationships as communities, each one formed by a different number of species of ticks, vertebrates, and borreliae that interact more among each other than with organisms of other communities. A visual inspection shows two prominent communities of interacting organisms (Nearctic at left, Eastern Palearctic at right) that are separated but connected to a central community, composed predominantly of species interacting with the tick *I. ricinus* (western Palearctic). The rest of the network consists of groups of organisms separated from these communities. Some communities, most notably those formed by Rf species, consist of a few species of ticks and vertebrates and lie completely separated from the main giant component of the network. The network also shows other relationships like *I. granulatus* or *I. turdus*, which are eastern Palearctic ticks but share hosts and species of *Borrelia* with *I. persulcatus*, a tick distributed from eastern Europe to Japan.

Of interest, the network reflects the associations of *B. burgdorferi* s.s. with both Palearctic and Nearctic ticks and vertebrates and includes it in an intermediate node, in the same cluster of *I. ricinus*. *Ixodes auritulus* is placed along the Nearctic cluster, as well as other non-*Ixodes* ticks (e.g., *Amblyomma americanum*, the vector of the Rf species *B. lonestari*) and the species of Argasidae transmitting Rf borreliae in the Nearctic, like *O. coriaceus*, *O. talaje*, or *O. hermsi*. However, *O. turicata* and *O. parkeri* (Nearctic) are clustered in a different community, together with *O. tholozani* and *B. persica* (non-Nearctic). *Ixodes pararicinus*, a neotropical species, is clearly separated into a different community. Other Rf species and their transmitting ticks are segregated into different communities, like *O. erraticus*, *O. tartakowskyi*, and *O. verrucosus*, together with *B. crocidurae*, *B. latyshewii*, and *B. caucasica*. These communities reflect an ecological distance between the Rf and the Lb stocks.

The Lb species circulate through dense, interconnected, and redundant networks of ticks and vertebrates, presumed to be developed to minimize competition (Estrada-Peña et al., 2015). Each group of Lb species is associated with a hierarchy of ticks and vertebrates. Additionally, each Lb species is associated with an assemblage of primary ticks that have the largest host range and with a group of secondary ticks, nested within the main community of vertebrates and Lb species. The vector status of most of these secondary tick species has never been studied, but they are strongly

linked to the main stream of vertebrates circulating different species of *Borrelia*. Therefore, the secondary ticks, which could be considered "specialists", tend to associate with generalist ticks, outlining a nested pattern of interactions.

We also approached the evolution of *Borrelia* through a phylogenetic analysis. We used phylogenetic trees of both ticks and vertebrates to calculate the Faith's phylogenetic distance (PD; Faith, 1992) of the partners on which each species of *Borrelia* has been recorded, summarized in Figure 2. The PD is a simple measure of the length of the branches in a phylogenetic tree linking any two taxa. A consistent association between the group of borreliae (Rf or Lb) and the PD of vertebrates in not straightforward. For instance, most Lb species have higher PD of vertebrates than Rf species. Furthermore, *B. miyamotoi* has higher PD of vertebrates than *B. lusitaniae*. Finally, from the data displayed in Table 1, the highest PD of vertebrates obtained for *B. burgdorferi* s.s. is consistent with records in a wide range of vertebrates of two continents. Additional conclusions can be drawn from the PD of ticks. Most Lb species are circulated by ticks with higher PD than Rf species. It would be reasonable to hypothesize that in addition to the well-demonstrated speciation of the Lb species along groups of vertebrates, a phylogenetic gradient of Lb species should be expected along the species of ticks. If the ecological pressures tend to minimize competition, the strains of Lb should change according to the vector.

We built phylogenies of the Rf and Lb species with the available *16S rRNA* and *ospA* and *ospC* sequences and MLST scheme. The MLST uses a series of eight housekeeping concatenated genes, providing more than 4000 base pairs for analysis. It is the *de facto* standard for identification of *Borrelia* (Margos et al., 2008; 2009). We assembled 717 sequences of 16S rRNA and 1654 strains of MLST genes to produce phylogenetic trees for which information about the vertebrate, the tick, and/or the geographical origin was available.

The summarized phylogenetic trees obtained from 16 rRNA, *ospA* and *ospC*, and MLST are included as Figures 3 and 4 and Supplementary material 1, respectively. Within the limits of data availability, these trees point to an evolutionary filter of some Lb species regarding the tick species with which they are associated. A visual inspection shows that the Lb species do not cluster around a species of tick, meaning that primary processes of speciation are driven by the vertebrate (as originally proposed by Kurtenbach et al., 2002), and only secondarily by the tick. In the case of the tree built on the 16S rRNA, clustering along ticks is observed for *B. miyamotoi*, with a high support value. The MLST-derived (Supplementary Material 1) tree confirms that (i) the strains of *B. miyamotoi* are associated with different species of ticks; (ii) there is a clear separation of *B. bissettiae* around *I. scapularis*, *I. spinipalpis*, and *I. pacificus*; (iii) *B. valaisiana* is restricted to *I. ricinus* and *B. bavariensis* to *I. persulcatus*, but with a branch associated with *I. ricinus*; (iv) there is

489 an unexpectedly large variety of strains of *B. yangtzensis*; and (v) there is a large gradient of *B.*
490 *afzelii* along *I. ricinus* and *I. persulcatus*.

491 These empirical results could be interpreted in several ways, and no data support one hypothesis
492 more than others. The different vertebrates on which ticks feed at a regional scale may act as a
493 secondary filter of the evolution of borreliae transmitted by *Ixodes*. This hypothesis has been
494 proposed by Vollmer et al. (2011): the biogeography of the vertebrate would impact the
495 phylogeography of *Borrelia* spp. For instance, both *B. garinii* (circulated through birds) and *B.*
496 *afzelii* (circulated through small mammals) show a different gradient of strain differentiation
497 because of the various local or regional movements of the main vertebrates in which they have been
498 recorded. However, results on the phylogeny of these species based on *ospA* (Figure 3B) clearly
499 support the linkage of these species of *Borrelia* to several species of ticks. We propose that this is
500 an example of species of *Borrelia* evolving along the lines imposed by different species of ticks
501 colonizing different environmental niches, as we already proposed in a previous study in which
502 clear association between MLST patterns, species of ticks and environmental niche was reported
503 (Estrada-Peña et al., 2016).

504 Available data support that ticks could exert a secondary pressure that produces a further selection
505 of *Borrelia*. It is interesting to note that the strains of *B. garinii* detected in *I. pavlovskyi* cluster near
506 the strains of *B. garinii* detected in *I. persulcatus* and are separated from those in *I. ricinus*. *Ixodes*
507 *pavlovskyi* and *I. persulcatus* have a parapatric distribution, the latter being sympatric in a small
508 portion of its range with *I. ricinus*. A further interpretation could be founded on the climate zonation
509 observed in the eastern Palearctic, following mainly a latitudinal gradient. Such zonation would
510 produce an obvious variation in the life cycle of the tick, which could further filter the strains of
511 bacteria.

512 The tick, as an ecosystem, could be the filter that exerts a secondary adaptation of *Borrelia*,
513 probably as a result of two not self-exclusive reasons:

514 a. The microbiome of each species of tick is exerting different pressures in the borreliae hosted
515 by the tick. Some laboratory experiments (Narasimhan et al., 2014) have demonstrated that
516 the presence of some common bacteria in the ticks can modify the behaviour of *Borrelia* in
517 the vector. On the other hand, increasing evidence points to the manipulation of the tick
518 molecular machinery by pathogens (Cabezas-Cruz et al., 2017), such as induction of
519 transcriptional reprogramming of infected cells, increasing tick fitness, and epigenetic
520 modulation of the tick gene expression, resulting in a potential transmission across
521 generations. Additional work and new methods focused on processing big data sets are
522 necessary to address this hypothesis.

b. Each species of tick is actually evolving different "strains" of borreliae, either because the finely tuned molecular adaptations of tick–*Borrelia* or because different climate conditions impact the life cycle of the tick. Previous experimental work shows that tick transmission imposes stochastic population bottlenecks on *B. burgdorferi* s.s. (Rego et al., 2014), supporting field data: ticks do not share strains of borreliae (Estrada-Peña et al., 2016, 2017). These bottlenecks could contribute to the variable prevalence of particular strains in geographically different areas (Rego et al., 2014). Taken together, data from laboratory observations, phylogenetic trees of *Borrelia* spp., and observations of the interactions among the three partners in the natural network are compatible with a multi-niche hypothesis for the evolution of *Borrelia* spp., which is evident in Lb species and at least in *B. miyamotoi*. Parts of the molecular machinery of *Borrelia* spp. (at least those revealed by MLST) are evolving by the variability of the ecological niche occupied by the ticks. Because several species of ticks can circulate the borreliae, the genetic variability of the bacteria reflects this adaptation to the "tick environment", wiping the strains that do not fit in a particular combination of tick+environment.

5. Conclusions

The bacteria of the genus *Borrelia* have a dual life cycle involving a tick vector and a vertebrate. Different evolutionary pressures have forced a strict dependence on both partners for the survival of *Borrelia*. These pressures drive the evolution of two groups of *Borrelia*, the Rf, transmitted by both argasid and ixodid ticks, and the Lb, circulated exclusively by ixodid ticks. To outline the probable evolution of *Borrelia*, we aimed to reconcile the molecular data available for *Borrelia* with the existing estimations of the evolution of ticks, geological land movements, and ecological relationships between ticks and vertebrates.

Available data suggest that the association of *Borrelia* with ticks is a very old one. The Rf species are the most ancient, probably evolving from tick symbionts, originally associated with the primitive pool of ticks. After the Argasidae–Ixodidae split occurring about 230–290 Mya, the restricted niche of Argasidae promoted the evolution of the Rf species associated with one species of tick and a few vertebrates. A few species remained associated to Ixodidae, circulating in a completely different ecological scenario. No Lb species are known to be transmitted by Argasidae; therefore, Lb species evolved after the split of the genus *Ixodes*.

The Lb species circulate through communities of generalist+specialist *Ixodes* spp. that act as a bridge between many host species. Different communities of interacting organisms use only one species of tick but share multiple vertebrates. This highly connected network of interactions

minimizes competition, exploiting a multiple niche and allowing molecular polymorphism and functional plasticity. Such a complex set of interactions produces a strain specialization of Lb species in which ticks could play the role of secondary filter, mainly driven by interactions with different ticks in the nested networks, a feature not paralleled in most Rf species.

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Legends for Figures

Figure 1. The hypothesis of evolution of the genus *Borrelia*, linking data on main geological events, land movements, and presumed evolution of ticks.

Parts of the figure are based on Mans et al. (2016), which includes the presumed date of important evolutionary events (column A), schemes of the movements of land masses (B), the assumed evolution of ticks (C) based on molecular clocks of mitochondrial DNA, and our proposed evolution of the genus *Borrelia*.

Figure 2. The phylogenetic diversity and species diversity of the vertebrates and ticks circulating *Borrelia* spp.

Phylogenetic diversity and species diversity for vertebrates (A) and ticks (B) circulating the species of *Borrelia* in the Lyme borreliosis group. Phylogenetic diversity and species diversity of the vertebrates (C) and ticks (D) circulating the species of *Borrelia* in the relapsing fever group. PD: phylogenetic diversity; Div: diversity of species.

Figure 3. 16S rRNA phylogenetic trees of Lb and Rf *Borrelia* and association with tick vectors.

Phylogenetic trees were built using Lb (A) and Rf (B) *Borrelia* 16S rRNA sequences. Sequences were collected from GenBank and aligned using MAFFT (Katoh and Standley, 2013). Redundant sequences (>99% identity) were removed using CD-HIT (Limin et al., 2012). The number of gap-free sites was increased using MaxAlign, which excluded poorly aligned sequences (Gouveia-Oliveira et al., 2007). The final alignments contained 209 and 160 nucleotide sequences and 955 and 1213 gap-free positions for Lb 16S rRNA and Rf 16S rRNA trees, respectively. The best-fit model of sequence evolution was selected based on Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC) implemented in Molecular Evolutionary Genetics Analysis (MEGA) 6 (Tamura et al., 2013). The Kimura-2 parameters (Kimura, 1980) model, which had the lowest values of cAIC and BIC, was chosen to build both 16S rRNA trees. Neighbour joining (NJ) and maximum likelihood (ML) methods, implemented in MEGA 6, were used to select the best topology explaining the evolution of each group of sequences. The NJ and ML topologies were nearly the same. To simplify the graphical representation, only the NJ topology is shown, and major branches containing similar sequences were collapsed. Bootstrap values (>60%) of clusters recovered with NJ and ML are shown. The full trees are provided in Newick format as supplementary material. When available, information on tick species that host the *Borrelia* was added next to the sequence.

Figure 4. *OspC* (A) and *ospA* (B) phylogenetic trees of Lb *Borrelia* and association with tick vectors.

Phylogenetic trees were built using *ospC* (A) and *ospA* (B) nucleotide sequences of Lb *Borrelia*. Sequences were collected from GenBank and aligned using MAFFT (Katoh and Standley, 2013). Redundant sequences (>99% identity) were removed using CD-HIT (Limin et al., 2012). The number of gap-free sites was increased using MaxAlign, which excluded poorly aligned sequences (Gouveia-Oliveira et al., 2007). The final alignments contained 386 and 188 nucleotide sequences and 427 and 626 gap-free positions for *ospC* and *ospA*, respectively. The best-fit models of sequence evolution were selected based on Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC) implemented in Molecular Evolutionary Genetics Analysis (MEGA) 6 (Tamura et al., 2013). The Generalised Time Reversible (GTR) (Tavaré, 1986) model, which had the lowest values of cAIC and BIC, was chosen to build the *ospC* and *ospA* trees. Neighbour joining (NJ) and maximum likelihood (ML) methods, implemented in MEGA 6, were used to select the best topology explaining the evolution of each group of sequences. The two topologies were nearly the same. To simplify the graphical representation, only the NJ topology is shown, and major branches containing similar sequences were collapsed. Bootstrap values of the clusters recovered with NJ and ML are shown. The full trees are provided in Newick format as supplementary material. When available, information on tick species that host the *Borrelia* was added next to the sequence.

Supplementary Material

Supplementary material 1. The phylogenetic tree of the phylogeny of the species of *Borrelia* based on the MLST scheme for which information exists about the species of ticks or vertebrates on which they were recorded. The tree is built with 8 in-house concatenated genes, aligned and free of gaps, as obtained from https://pubmlst.org/borrelia/ (accessed, August 2017). Neighbour joining (NJ) and maximum likelihood (ML) methods, implemented in MEGA 6, were used to select the best topology explaining the evolution of each group of sequences. The two topologies were nearly the same. To simplify the graphical representation, only the NJ topology is shown. The label of each sequence is formed by a sequential number for internal use only, the species of *Borrelia*, the species of tick or vertebrate, and the MLST strain number, according to the standards of the method (Margos et al., 2008).

Supplementary material 2. The Newick files of the 16S sequences of *Borrelia* spp. available in GenBank with information on the tick or the vertebrate of record. Species included in the groups Rf or Lb are provided separately.

Supplementary material 3. The Newick files of the *ospA* and *ospC* sequences of *Borrelia* spp. available in GenBank with information on the tick or the vertebrate of record.

Supplementary material 4. The network of interactions among species of *Borrelia*, ticks, and vertebrates. The network is based on published reports, approximately from the year 1980. All methods used to build the network already have been published (Estrada-Peña et al., 2015). The network contains only the names of the species of ticks and *Borrelia* to improve the readability. A complete version of the same drawing, with labels for every organism included, is available as Supplementary Material 4. In this network, circles (nodes) are interacting organisms, and links mean for interactions among. The colours of the nodes and links are random and indicate a cluster or communities of organisms that interact more among themselves than with the rest of the nodes. The topology of the network reflects the relationships (interactions) among the clusters. The size of each node is proportional to its centrality in the network, and the size of its label is proportional to its weighted degree, a weighted measure of the number of reports of the organism.

Supplementary material 5. The network of interactions among species of *Borrelia*, ticks, and vertebrates. The network is based on published reports, from approximately 1980. All methods used to build the network already have been published (Estrada-Peña et al., 2015). The network contains labels for every organism included and can be zoomed in to improve readability. In this network, circles (nodes) are interacting organisms, and links indicate interactions. The colours of the nodes and links are random and indicate clusters or communities of organisms that interact more among themselves than with the rest of the nodes. The topology of the network reflects the relationships

(interactions) among the clusters. The size of each node is proportional to its centrality in the network, and the size of its label is proportional to its weighted degree.